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Research Article 研究报告

Effect of genistein on MRSA efflux protein expression

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Abstract: [Objective] To study the effect of genistein on methicillin-resistant *Staphylococcus aureus* (MRSA) efflux proteins. **[Methods]** Combined drug sensitivity test was used to detect the effect of genistein on the sensitivity of MRSA to ciprofloxacin. The isobaric tags for relative and absolute quantitation (iTRAQ) technology were used to detect the change of bacterial cell protein expression after genistein was applied to MRSA41577. The bioinformatics method was used to analyze the significantly different proteins. qPCR and Nile Red efflux assay were used to explore the mechanism of drug resistance-related proteins mediating bacterial resistance. **[Results]** Combined drug sensitivity test showed that genistein enhanced the sensitivity of MRSA to ciprofloxacin. A total of 129 significantly different proteins were detected by iTRAQ technology, including 60 proteins with up-regulated expression and 69 proteins with down-regulated expression. Bioinformatics analysis showed that there were about 14 proteins related to bacterial resistance, of which PstB, PstS and other proteins mainly mediated bacterial resistance through the active efflux system. qPCR results showed that compared with the control group, PstB and PstS gene expression decreased by 51.6% and 78.6%, respectively. Nile Red efflux assay showed there was a competitive relationship between genistein and Nile Red, so that genistein was a competitive inhibitor of MRSA41577. **[Conclusion]** Genistein reverses bacterial resistance by reducing mRNA expression of the efflux genes *pst*B and *pst*S, and affected the expression of efflux proteins PstB and PstS of MRSA41577. Besides, genistein is also a competitive efflux inhibitor of MRSA41577 and plays antibacterial effect by competing with substrates for efflux, which makes antibacterial drugs stay in the bacteria.

Keywords: MRSA, efflux protein, protein differential analysis, iTRAQ

Infectious diseases caused by MRSA spread all over the world, and MRSA is one of the pathogens which cause the highest incidence of hospital infection and community infection[1–4], which seriously threatening human health and global public health security[5]. MRSA has become a focus of world attention due to its multiple drug resistance, complex drug resistance mechanism,

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high infection rate and high fatality rate[6-9]. Previous studies have shown that the active efflux system and biofilm formation of MRSA are the main reasons leading to multiple drug resistance[10–14]. Efflux pump inhibitor (EPIs) is active to inhibit MRSA efflux system and reverse its drug resistance. In the early stage, from 8 plant monomer compounds, genistein, an efflux pump inhibitor with better effect, was screened out by combined drug sensitivity test, double plate method and fluorescence spectrophotometry. Genistein is a naturally occurring isoflavone that has been shown to have anti-inflammatory, antibacterial, anti-cancer effects[15]. However, the specific regulatory mechanism of genistein on the active efflux system is still unclear. It has been shown that bacterial drug resistance caused by the active efflux system is related to the bacterial drug resistant proteins. These efflux proteins can pump out antimicrobial agents that are unfavorable to bacteria, enhance the survival ability of bacteria under the pressure of antimicrobial drug selection, and enable bacteria to show multi-drug resistance[16]. Also, studies have reported that related proteins in phosphate specific transport systems, such as PstB and PstS, are also related to bacterial resistance. Therefore, in this paper, iTRAQ technology was used to detect the changes in the expression level of bacterial protein after the action of genistein on MRSA, and bioinformatics methods were used to analyze the mechanism of drug resistance-related proteins inhibiting the active efflux system of MRSA.

1 Materials and methods

1.1 Materials

MRSA41577 was obtained from the Dalian Municipal Central Hospital (Dalian, Liaoning, China). *S. aureus* ATCC25923 was provided by the Chinese Medical Culture Collection Center, National Center for Medical Culture Collections (Beijing, China; www.cmccb.org.cn/cmccbnew). Nile red and genistein were purchased from Novozymes Biotechnology Co., Ltd. (Shenyang, China). Primers and Real-Time PCR reagents were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Spin Column Bacteria Total RNA Purification Kit was purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

1.2 Combined drug sensitivity test to detect genistein restoring the sensitivity of MRSA to ciprofloxacin

Antimicrobial activities of genistein and ciprofloxacin were determined according to a standardized broth microdilution method (Clinical and Laboratory Standards Institute (CLSI) document M27-A2)[17]. Add 100 μL of bacterial suspension (10^5 CFU/mL) and 10 μL of ciprofloxacin (CIP) with different concentrations (final concentrations: 8–256 μg/mL) in a 96-well plate. Then, 10 μL of genistein at different concentrations (final concentrations: 64–16 μg/mL) were added. The plates were incubated at 37 °C and 40×*g* for 24 h, then aqueous 2,3,5-triphenyl tetrazolium chloride (TTC) solution (0.2%, 20 μL) was added to indicate the viability of bacteria. After 4 h of incubation in the dark, the results were observed. The test was carried out in triplicate with genistein as the control group and 20 μg/mL reserpine as the positive control. Red means bacterial growth, transparent means no bacterial growth. MIC values were determined as the lowest concentration of the ciprofloxacin capable of inhibiting visible microorganism growth.

1.3 Determination of the effect of genistein on MRSA41577 protein expression

The MRSA41577 broth cultured at 37 °C overnight was inoculated to a 20 mL fresh LB liquid medium for 16 h to log phase at a ratio of 1%. Then, genistein was added as a final concentration of 32 μg/mL (Our previous results showed that 32 μg/mL genistein had the best effect on restoring

the drug resistance sensitivity of MRSA41577), and continue to incubate at 37 °C for 16 h to log phase with no drug group as a blank control. The two groups of bacteria cultured grown up to log phase were centrifuged at 4000×*g* for 5 min at 4 °C to collect the cells. Wash 3 times with pre-cooled phosphate buffer solution (pH 7.4), mix 200 μg of the lysate (20 mmol/L Tris, pH 8.0, 50 μg/mL lysostaphin, 30 U nuclease) per 100 μg cells and incubate at 37 °C for 1 h. Add 700 μL of protein lysate (2 mol/L thiourea, 7 mol/L urea, 4% CHAPS, 0.5% ampholyte) and incubate at room temperature for 3 h, then collect the supernatant by centrifugation at 2000×*g* at 4 °C. The iTRAQ technique was used to detect the differential protein between the blank control group and the experimental group. This experiment was carried out by Dalian Sato Biotechnology Co., Ltd.

1.4 Analysis of the effect of genistein on MRSA41577 protein expression

The iTRAQ method was used to screen for differentially expressed proteins. The peptides were tagged with iTRAQ, SCX graded, desalted and analyzed by lc-msms mass spectrometry. About 80 μg sample peptides were taken from each group and labeled according to the instructions of the AB company kit: iTRAQ Reagent-8plex Multiplex Kit (AB SCIEX). About 36 fractions of eluted fractions were collected and combined into 6 fractions according to the SCX chromatogram, and the C_{18} Cartridge was desalted after lyophilization. The raw data of the mass spectrometry analysis were RAW files. The software Mascot 2.2 and Proteome Discoverer 1.4 were used to identify and quantify the library. The database used is the Uniprot-Staphylococcus-aureus-123969. fasta database. Gene Ontology Analysis (Quick Go) was used to analyze the biological processes involved in the differential proteins, cell composition and molecular functions; the differential proteins involved in the analysis were analyzed by the Kyoto Encyclopedia of Genes and Genomes (KEGG); Functional protein association networks (STRING) was used to analyze the network pathways between differential proteins. The functional annotation of the differential proteins was combined to find proteins related to the efflux of MRSA41577.

1.5 Assessment of the effect of genistein on MRSA41577 *pst*B and *pst*S genes expression levels

MRSA41577 (10^5 CFU/mL) was cultured in the absence or presence of 32 μg/mL genistein at 37 °C to the log phase. The total RNA was extracted using Total RNA Purification Kit, and the template cDNA was synthesized by a two-step inversion method. cDNA was used as the template to conduct quantitative gene amplification according to *pst*B gene and *pst*S gene primers, and 16S rRNA was used as the internal reference gene to determine the effect of genistein on gene expression by real-time fluorescence qPCR. The primer sequence is as follows:

*pst*B (F: 5′-TTGCAGCGCCACGTAATGAT-3′; R: 5′-AGGTCCATCTGGTTGTGGCA-3′);

*pst*S (F: 5′-CAGCCGCAGCATCTACATCTCC -3′; R: 5′-ACCACCAACGGCATACACATTAG C-3′).

In the qPCR instrument, all genes were amplified under the following conditions: 95 °C 30 s; 95 °C 5 s, 53 °C 30 s, 40 cycles; 95 °C 15 s, 60 °C 30 s, 95 °C 15 s.

1.6 Determination of competition between genistein and MRSA41577 efflux substrates[18]

Nile red is a lipophilic fluorescent dye which can emit strong fluorescence binding to lipid substances (such as lipids on cell membranes) when it enters bacterial cells. Measuring the intensity of fluorescence can reflect the content of Nile Red in cells. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) is an efflux pump energy inhibitor that causes cells to lose their energy source. Therefore,

Nile Red can attach to bacterial cells to express the corresponding fluorescence intensity.

First, we investigated the highest concentration of fluorescence intensity of cells attached to Nile red. MRSA41577 was inoculated in a liquid medium at 2% inoculum and incubated at 50×*g* overnight at 37 °C for 14–16 h (OD_{660} normally reached a plateau). The cells were centrifuged at 1500×*g* for 10 min at room temperature and centrifuged and resuspended once the bacterial colonies were centrifuged with potassium phosphate buffer (pH 7.0) (PPB) containing a final concentration of 1 mmol/L $MgCl_2$ at 20 mmol/L. Adjust OD_{660} to 1.0 with PPB. Divided into 10 small test tubes, room temperature for 15 min. Then add CCCP at a final concentration of 10 μmol/L to the tube. Take the mother liquor as 5 mmol/L nylon, diluted to 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 μmol/L concentration gradient in turn into the test tube. 50×*g* at 37 °C for 3 h. Room temperature for 60 min, 1500×*g* centrifugation 5 min, discard the supernatant, the liquid on the wall with cotton all dry. 0.2 mL of cell suspension plus 1.8 mL of PPB was added to the quartz cup with a gap width of 10 nm, an excitation wavelength of 552 nm and an emission wavelength of 636 nm. The fluorescence intensity of 100s cells was measured.

After determining the optimum fluorescence intensity of Nile red. Take 10 mL overnight incubated MRSA41577 and ATCC25923 bacterial solution, centrifuged at 1500×*g* for 10 min at room temperature, 20 mL of the final concentration of 20 mmol/L PPB, centrifuged and resuspended again. Adjust OD_{660} to 1.0 with PPB. At room temperature for 15 min, MRSA41577 bacterial solution by adding 5 μmol/L CCCP, ATCC25923 by adding 5 μmol/L CCCP. After 15 min resting, packing, adding different concentrations of genistein, and then resting for 15 min. The final concentration of 4 μmol/L Nile red was added and cultured at 37 °C for 3 h with shaking at 37 °C. Room temperature for 60 min, 1500×*g* centrifugation 5 min, discard the supernatant, the liquid on the wall with absorbent cotton all dry. Each group of cells was resuspended in 2 mL of PPB containing a final concentration of 0, 16, 32, 64 μg/mL of genistein and 10, 20 μg/mL of reserpine. The 0.2 mL cell suspension was rapidly added to 1.8 mL of PPB and added to a quartz cup. The fluorescence wavelength was 10 nm, the excitation wavelength was 552 nm, and the emission wavelength was 636 nm. The fluorescence intensity of 100s cells was measured. After adding 100 μL of 1 mol/L glucose, the detection was continued for 200 s. Record the change in fluorescence intensity.

1.7 Statistical analysis

Data were analyzed by using SPSS 13.0 and Microsoft Excel 2010 software. One-way analysis of variance, using the Dunnett Multiple Comparison test, was carried out on data obtained from three independent assays performed in duplicate for each sample. Levels of statistical significance at P<0.05 and P<0.01 were used.

2 Results

2.1 Genistein enhances ciprofloxacin sensitivity of MRSA

The results of the combined drug sensitivity test showed that genistein could significantly enhance ciprofloxacin sensitivity of MRSA (Table 1). Among them, 32 μg/mL genistein combined with ciprofloxacin can reduce the MIC of ciprofloxacin by MRSA from 128 μg/mL to 32 μg/mL, a 4-fold reduction. The effect is consistent with the positive control reserpine.

Table 1. The synergistic effect of genistein and CIP

Concentration/(μg/mL)	256	128	64	32	16	8
Ciprofloxacin (CIP)	–	–	+	+	+	+
CIP+16 genistein	–	–	–	+	+	+
CIP+32 genistein	–	–	–	–	+	+
CIP+64 genistein	–	–	–	–	+	+
CIP+20 reserpine	–	–	–	–	+	+

+: bacterial growth; –: no bacterial growth.

2.2 Determination of the effect of genistein on MRSA41577 protein expression

A total of 1312 proteins and 6553 peptides were detected. There were 129 proteins express significant differences (difference protein P<0.05, the difference in appearance between groups was more significant than 1.2 or less than 0.833), including 60 up-regulated proteins and 69 down-regulated proteins.

2.3 Analysis of the effect of genistein on MRSA41577 protein

The bioinformatics analysis results were as follows: The GO analysis included the analysis of biological processes involved in differential proteins, cell composition, and molecular function. The results of GO analysis (Figure 1) showed that the 129 differential proteins affected by genistein were involved in 13 biological processes, eight cellular components, and six molecular functions. The biological processes in which these differential proteins are involved are mainly metabolic processes, accounting for 80%, followed by single organism processes (58%) and cellular processes (65%). The distributions in cell locations are cell types (46%), cell component category (44%), cell membrane (22%), and cell membrane component (18%).

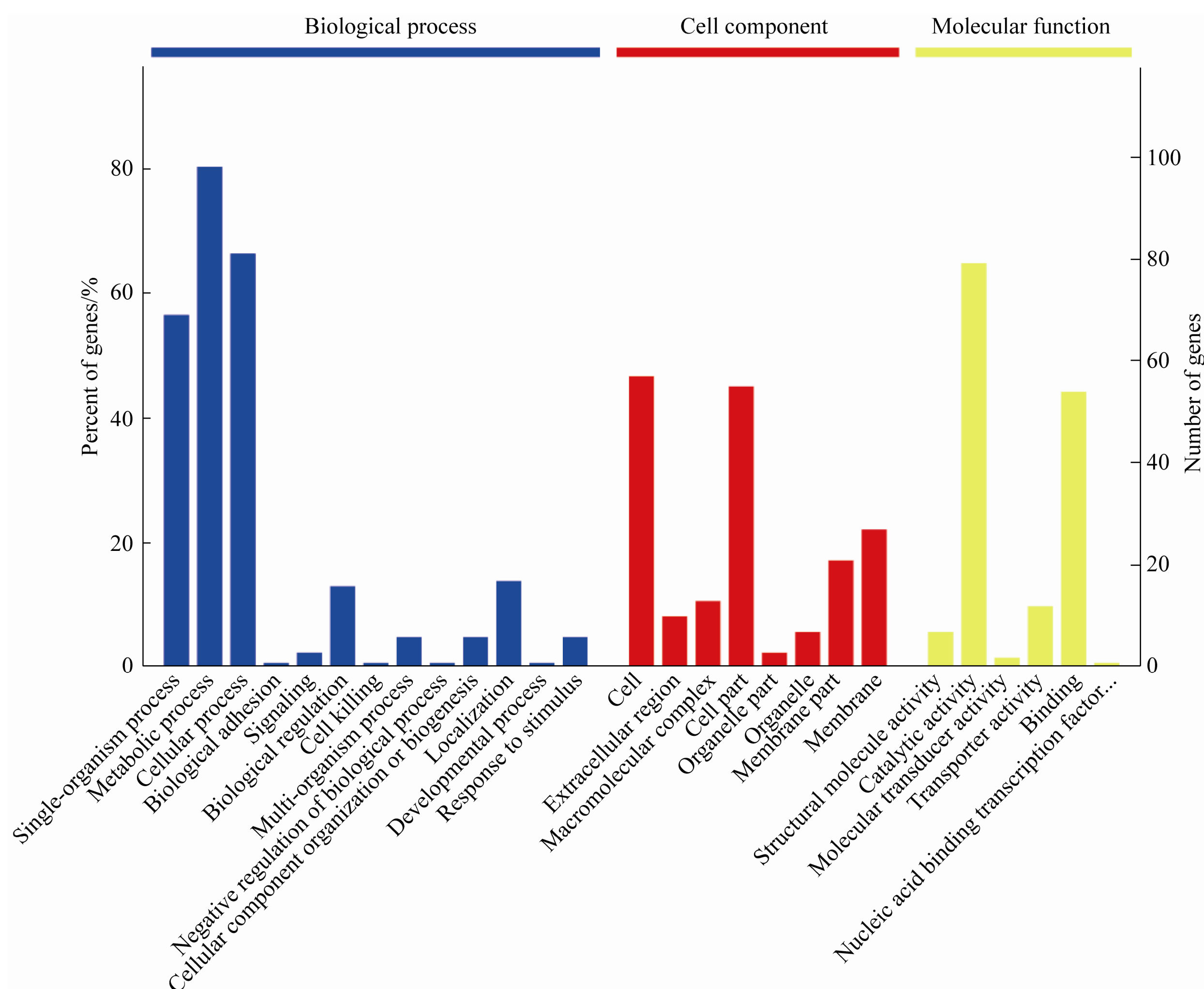

Figure 1. Gene ontology analysis. GO annotation of differentially regulated protein (ratio: >1.2 or <0.833) functions. *Y*-axis represented the number of identified proteins in each GO category.

The differential proteins perform molecular functions, including 63% of catalytic activity, 44% of binding molecules, and 10% of transport activity. Based on the results of comprehensive GO analysis, it is speculated that the components present in the cell membrane of the cell and the differential proteins that perform the transport activity function may be related to the active efflux system of MRSA41577. The KEGG pathway database analysis results (Figure 2) showed that the 129 differential proteins affected by genistein involved four significant pathways: Metabolism, Genetic Information Processing, Environmental Information Processing, and some unknown paths. GO analysis speculates that proteins involved in membrane transport may be included in active efflux systems. In the membrane transport pathway represented by the CA, there are eight differential proteins in the ABC transport pathway. There is a differential protein in the phosphotransferase system pathway; there is a differential protein in the bacterial secretion pathway, a two-component transport system. There are seven differential proteins in the path, so it is speculated that these 17 differential proteins may be related to the active efflux system of MRSA41577. STRING analyzes the network pathways of the interactions between the differential proteins (Figure 3). Each circle represents a protein, and the marginal distance of the ring depicts the correlation with the predicted protein function.

The above biological information analysis results showed that out of the 129 differential proteins, 19 proteins related to MRSA41577 resistance were identified. Five differential proteins were associated with disease infection, 14 differential proteins are involved in substrate-specific transmembrane transport, including six up-regulated and eight down-regulated proteins. It was shown that PstB, PstS, etc. mainly mediate bacterial resistance through active efflux systems.

2.4 Effect of genistein on the expression of pstB mRNA and pstS mRNA

The results showed that compared with the control group, the mRNA expression levels of the down-regulated proteins PstB and PstS were significantly decreased (Figure 4), and the gene expression levels were reduced by 51.6% and 78.6%, respectively (P<0.01).

2.5 Competition between genistein and MRSA41577 efflux substrate

The results showed that the optimal concentration of Nile red attached to MRSA41577 bacterial cells was 4 μmol/L, and the fluorescence intensity was the highest (Figure 5). With the increase of strength, the fluorescence intensity is reduced, presumably may be spontaneously quenched in the cell membrane. So we chose 4 μmol/L for the experimental concentration of Nile red. Nile red is a lipophilic dye when it is in aqueous solution, the fluorescence is very weak, but in a non-polar environment, it will issue a vigorous fluorescence intensity. The results showed that the fluorescence intensity of the ATCC25923 was lower, and the fluorescence intensity of the MRSA41577 was higher (Figure 6). The fluorescence intensity of the MRSA41577 was higher indicates the presence of the efflux pump and quickly pump the Nile red out of the body. Similar to the positive control drug reserpine, the treatment of MRSA41577 with genistein increased the fluorescence intensity of Nile red in a dose-dependent manner. This result indicates that genistein inhibits the efflux of Nile red in a dose-dependent way in MRSA41577 cells (Figure 7). Reserpine is a competitive efflux pump inhibitor that competes with the efflux substrate to bind to the active site of the effluent pump, allowing the efferent substrate to remain in the cell. Similar to reserpine, genistein inhibits Nile red efflux by MRSA41577 cells. It is hypothesized that there is a competitive relationship between the effluent lignin and Nile red, a competitive inhibitor of MRSA41577.

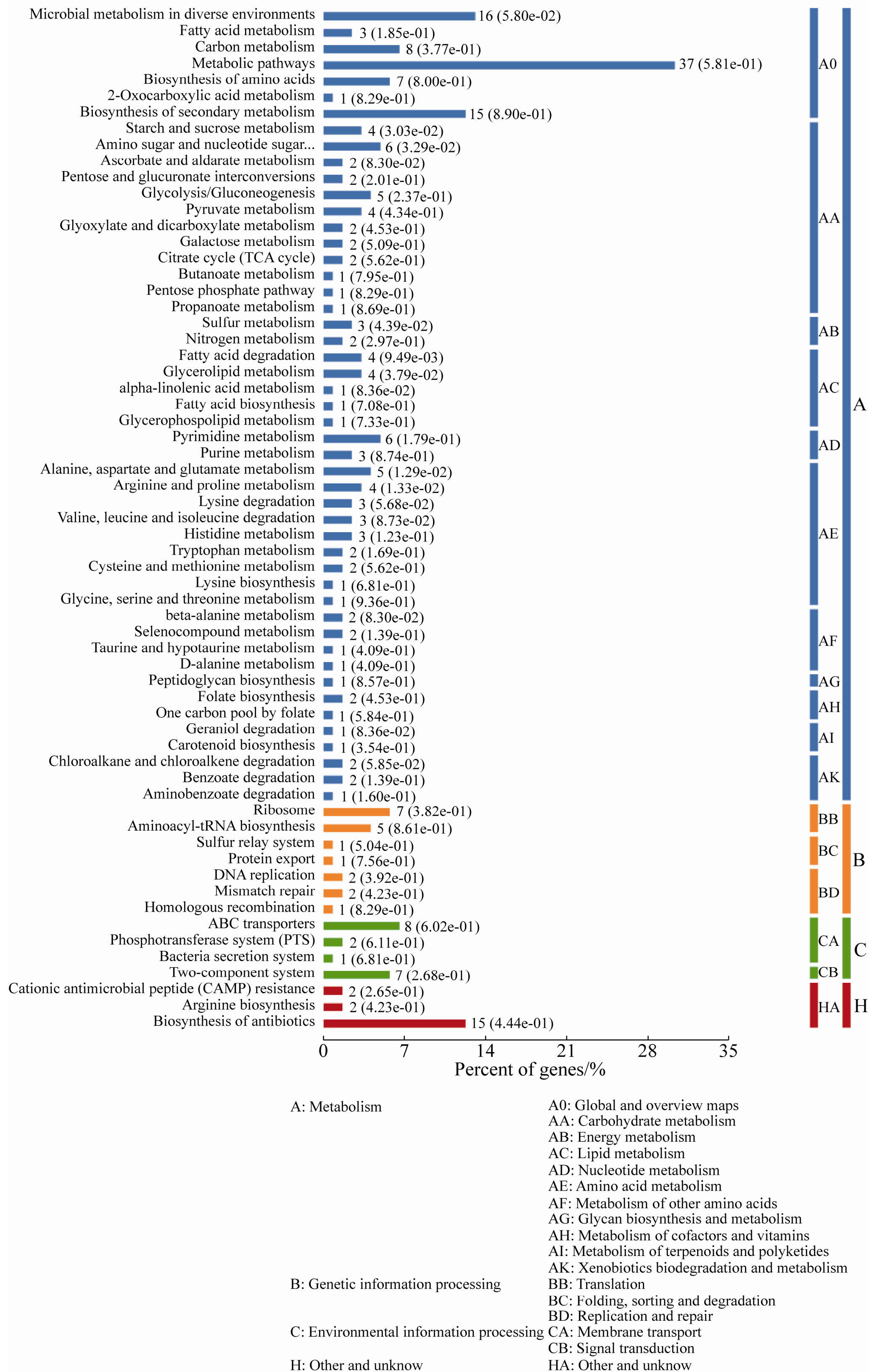

Figure 2. Proteins involved in KEGG pathways.

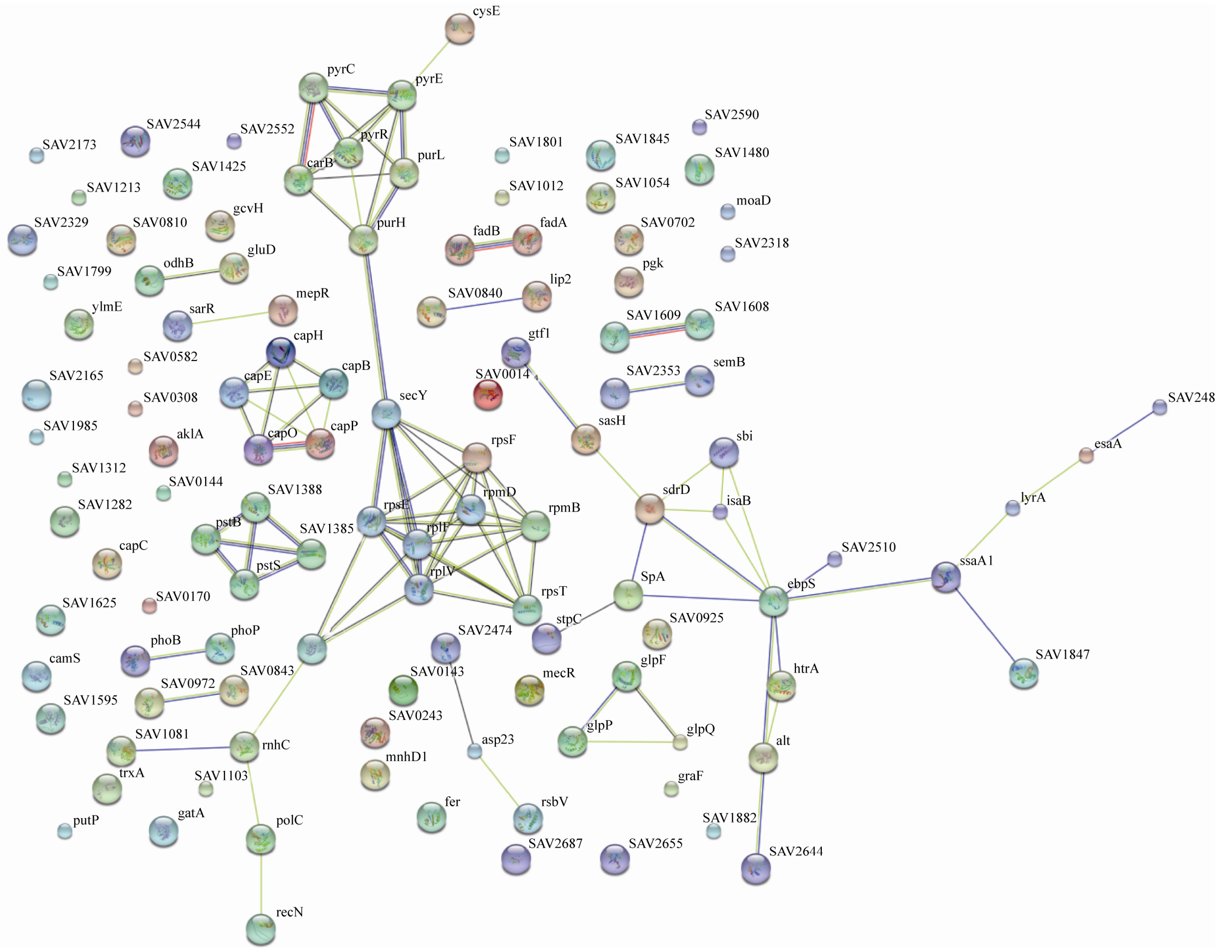

Figure 3. Protein-protein interaction analysis. The network of significantly differentially expressed proteins (ratio: >1.2 or <0.833 fold) was analyzed by String. Small nodes represent protein of unknown 3D structure; large nodes represent some 3D structure is known or predicted. Colored nodes represent query proteins and first shell of interactors; white nodes represent second shell of interactors. The blue lines represent database evidence; the purple lines represent experimental evidence; yellow lines represent text mining evidence; the black lines represent coexpression evidence; and green lines represent neighborhood evidence.

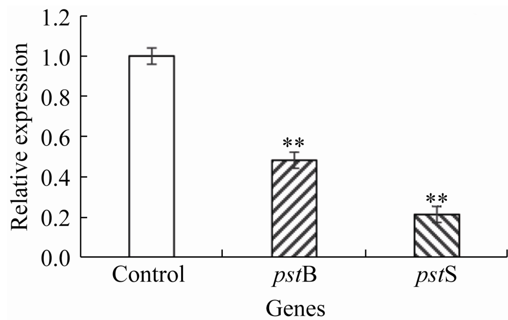

Figure 4. Effects of genistein on the expression of *pst*B, *pst*S genes of MRSA41577. *: P<0.05, compared with control; **: P<0.01, compared with control.

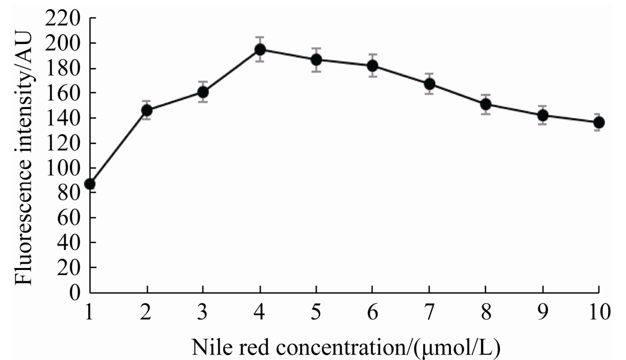

Figure 5. The optimal concentration of Nile red.

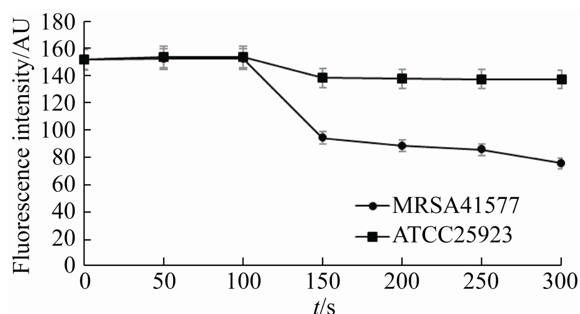

Figure 6. Nied red efflux in MRSA41577 and ATCC25923.

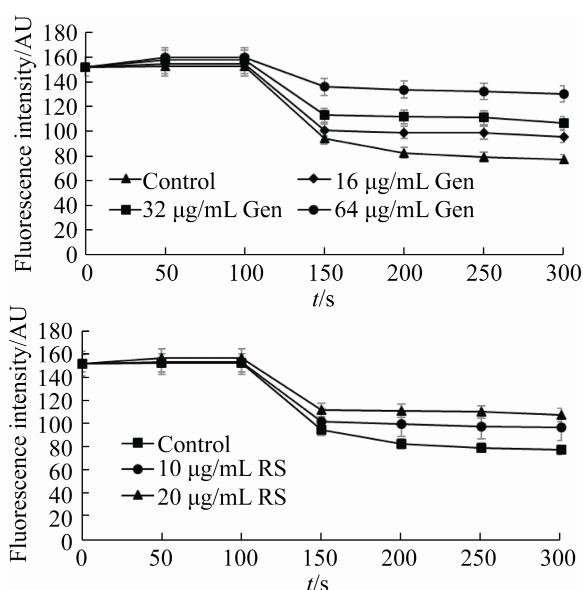

Figure 7. Dose-dependent inhibition of Nile red efflux by genistein (Gen) and reserpine (RS).

3 Discussion

Methicillin-resistant Staphylococcus aureus (MRSA) has a multi-drug resistance to various antibacterial agents, which severely affects human life and health[19–20]. Studies have shown that the active efflux system of MRSA is one of the main reasons for its multi-drug resistance[21]. The active efflux system is associated with drug resistance proteins. The difference in expression of MRSA bacterial proteins after the teeatment of genistein showed that 129 proteins were detected, including 60 proteins with up-regulated expression and 69 proteins with down-regulated expression. Further bioinformatics analysis obtained 14 drug resistance-associated proteins, among which PstB, PstS and other proteins can mediate bacterial resistance through an active efflux system. The expression levels of PstB and PstS protein genes were detected by qPCR method, and the results showed that genistein could inhibit the transcription and expression of bacteria gene *pst*B and *pst*S *in vitro*, and decrease the content of efflux protein PstB and PstS. The results of the Nile Red efflux assay showed that genistein competed with the substrate for efflux, ensuring the accumulation of drug in MRSA41577 to carry out bacteriostatic effect as a competitive EPI.

Also, AVI Neznansky et al. showed that when the *pstS* gene was knocked out, the ability to form a biofilm (BF) was significantly reduced or the expression of invasive genes such as *hilA* was reduced to alleviate pathogenic bacteria infection to host cells[22–23]. Therefore, PstS protein can also mediate MRSA resistance by affecting the formation of bacterial BF.

In summary, genistein can inhibit the expression of efflux protein PstB and PstS protein. At the same time, it can inhibit the active efflux system of MRSA41577 by competing with antibacterial drugs and allowing antibacterial drugs to remain in the bacteria to exert antibacterial action, thereby reverse the multi-drug resistance of MRSA.

Competing interests

The authors declare that they have no competing interests.

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染料木素对 MRSA 外排蛋白表达的影响

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摘要：【目的】研究染料木素对耐甲氧西林金黄色葡萄球菌(MRSA)外排蛋白的影响。【方法】通过联合药敏实验检测染料木素影响 MRSA 对环丙沙星的敏感性；利用等重同位素多标签相对定量蛋白质组学(iTRAQ)技术，检测染料木素作用 MRSA41577 后菌体蛋白表达量的变化；通过生物信息学方法对差异显著的蛋白进行系统分析；通过 qPCR 和尼罗红外排实验，探讨耐药相关的蛋白介导细菌耐药的作用机制。【结果】联合药敏实验结果显示，染料木素能增强 MRSA 对环丙沙星的敏感性；通过 iTRAQ 技术检测到差异显著蛋白共有 129 个，包括 60 个表达上调的蛋白和 69 个表达下调的蛋白；生物信息学分析结果显示，与细菌耐药相关的蛋白约有 14 个，其中，通过主动外排系统介导细菌耐药的蛋白主要有 PstB、PstS 等；qPCR 结果显示，与对照组相比，PstB、PstS 的基因表达量分别下降了 51.6%和 78.6%；尼罗红外排实验结果显示，染料木素与尼罗红之间存在竞争关系，为 MRSA41577 的竞争性抑制剂。【结论】染料木素可通过降低 MRSA41577 外排基因 *pstB* 和 *pstS* 的 mRNA 表达量，进而影响 PstB、PstS 外排蛋白的表达来逆转细菌耐药；此外，染料木素还是 MRSA41577 的竞争性外排抑制剂，可通过与底物竞争外排的方式，使抗菌药物留在菌体内发挥抗菌作用。

关键词：耐甲氧西林金黄色葡萄球菌，外排蛋白，蛋白质差异分析，同位素标记相对和绝对定量

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